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13. ABSTRACT *(Maximum 200 words)*

Compared to the activation state of Ras in normal human breast tissue and in fibrocystic breast tissue, we found Ras activation levels to be significantly increased in 11 of 20 human breast cancers. The increased Ras activation in the 11 breast cancers correlated with expression of the epidermal growth factor (EGF) receptor but did not correlate with expression of the ErbB-2 receptor, the c-FMS receptor, or a constitutively-activated EGF receptor; in none of the cancers was there evidence for an activating mutation in the *ras* gene. In the tumors with increased Ras activation, there was a marked increase in mitogen-activated protein (MAP) kinase activity indicating that the increased Ras activation was physiologically significant. This work shows for the first time that Ras is activated in a significant number of breast cancers and that the mechanism of activation appears to be through increased expression of a growth factor receptor. Moreover, this work lays the groundwork for the future treatment of human breast cancers with inhibitors of Ras and/or of the Ras/MAP kinase pathway; several of these agents are currently in Phase I and II Clinical Trials.

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INTRODUCTION

Ras is an important cellular switch which relays growth-promoting signals from the plasma membrane to the nucleus (1). It is in an activated state when bound to GTP and in an inactivated state when bound to GDP. One of the major "downstream" effectors of Ras is mitogen-activated protein (MAP) kinase which translocates to the nucleus on activation and phosphorylates, and thereby activates, several transcription factors involved in regulating cellular growth and differentiation (2).

Many growth factors activate Ras on binding to their respective receptors and two of the receptors which signal through Ras are the epidermal growth factor (EGF) and the ErbB-2 receptor (3). These two receptors are each overexpressed in 20-50% of human breast cancers and their overexpression generally correlates with a poor clinical prognosis (4-7). Genetic mutations in the *ras* gene occur in $<5\%$ of breast cancers and this has led to the notion that Ras does not play an important etiologic role in the development of this cancer (8,9). However, because of overexpression of the EGF and ErbB-2 receptors in breast cancer, we hypothesized that Ras might be in a highly activated state in a significant number of breast cancers. We recently devised a method to assess the activation state of Ras in human tissue (10) and in this project we have applied this technology to assessing Ras activation levels in human breast cancers. Determining the activation state of Ras in breast cancer is important for two reasons. First, it provides information about the basic biology and pathogenesis of this tumor. Second, and perhaps more importantly, it may lead to novel treatment strategies for this cancer because a large number of Ras inhibitors are currently being tested in Phase I and II Clinical Trials; in cultured cell systems and in animal models, these agents are only effective in tumors expressing activated Ras.

BODY

In the original grant application we proposed to analyze the activation state of Ras and of MAP kinase in 60 breast cancers, 10 normal breast samples and 50 fine needle aspirates of breast lesions during the three year tenure of the grant. During the first year of the grant, we have assessed the activation state of Ras and of MAP kinase in 20 breast cancers, seven normal breast samples, four samples with fibrocystic disease, and 15 fine needle aspirates.

We are writing a manuscript (a preliminary draft of the manuscript is in the appendix) which describes the results with all of the samples except the fine needle aspirates; these latter data will be published elsewhere. We found Ras activation levels of $5.5 \pm 1.9\%$ (mean $\pm$ S.D.) in the seven normal breast samples (Fig. 1, page 7) which are very similar to the Ras activation levels we have found in other normal human cells and tissues including ovaries, brain, peripheral nerves, and peripheral blood leukocytes ((10-12) and unpublished data). The mean Ras activation levels in the fibrocystic tissue were very similar to that found in the normal breast tissue (Fig. 1). Ras activation levels in the 20 breast cancers ranged from 1.5% to 29% with nine of the cancers having Ras activation levels within two standard deviations of the mean of the normal samples; these nine cancers were combined together as Group A. The remaining 11 cancers with Ras activation levels that exceeded two standard deviations beyond the mean of the normal samples were combined together as Group B (Fig. 1). There was no difference between the Group A and Group B cancers in terms of size, grade, histopathology, percent cells in S

phase or chromosomal ploidy; similarly, there were no differences between the patients in these two groups in terms of age, clinical stage, etc. The elevated Ras activation levels in the Group B cancers could not be attributed to elevated Ras activation levels in the breast tissue of these patients because in two patients we also analyzed Ras activation levels in normal (or fibrocystic) tissue distant from the tumor and we did not find Ras activation levels to be elevated in these other samples. In addition, the increased Ras activation in the tumors was not secondary to an activating mutation in the *ras* gene because we assessed each sample for a K-*ras* codon 12 mutation by polymerase chain reaction/allele-specific restriction enzyme digestion and found no evidence of a mutation in any of the samples, benign or malignant (as mentioned earlier, mutations in the *ras* gene are rare in breast cancer and when they occur, >95% are in K-*ras*, codon 12). Thus, in slightly more than half of the breast cancers, Ras activation levels were clearly elevated and the increased Ras activation in these tumors was not secondary to a mutation in the *ras* gene.

The most likely cause for increased Ras activation in the Group A cancers was from increased expression of a growth factor receptor that signals through Ras and we, therefore, assessed the samples for expression of the EGF and ErbB-2 receptors, two receptors which are known to be overexpressed in breast cancer. None of the normal breast samples and none of the cancers in Group A expressed the EGF receptor while all but one of the analyzed cancers in Group B expressed this receptor (Fig. 2, page 7); one of the fibrocystic samples expressed the EGF receptor, but this sample was from the breast of one of the patients with a Group B cancer. Thus, there was a rather remarkable correlation between elevation of Ras activation levels and expression of the EGF receptor. There was little correlation between Ras activation levels and expression of the ErbB-2 receptor because three of the seven normal breast samples, one of the four fibrocystic samples and three of the nine Group A cancers expressed this receptor as did eight of the 11 cancers in Group B (Fig. 3, page 7). Interestingly, the one Group B cancer which did not express the EGF receptor did express the ErbB-2 receptor (compare Figs. 2 & 3). We also assessed the samples for expression of a truncated constitutively-active EGF receptor and for expression of the c-FMS receptor (the latter receptor also signals through the Ras/MAP kinase pathway); both of these receptors have been reported to occur in breast cancers (13,14), but we did not find expression of either of these receptors in any of the samples analyzed. This may be because previous antibodies used cross-reacted with some other antigen, for example, an antibody against the truncated EGF receptor could cross-react with the normal EGF receptor. Overall, the data suggest that Ras may be activated in the Group B cancers because the tumors express high levels of the EGF receptor.

Because MAP kinase is a major downstream effector of Ras, we assessed the activation state of MAP kinase in the breast samples that we studied. We found similar levels of MAP kinase activity in the normal breast samples and the fibrocystic samples (Fig. 4, page 8). Although MAP kinase activity was higher in the Group A breast cancers than in the normal samples (Fig. 4), this difference did not reach statistical significance because of relatively large standard deviations within the data. However, MAP kinase activity was almost ten times higher in the Group B cancers than in the normal breast samples and this difference in enzyme activity was significantly different (Fig. 4). Thus, there was a good correlation between the level of Ras activation in a tumor and the degree of MAP kinase activity.

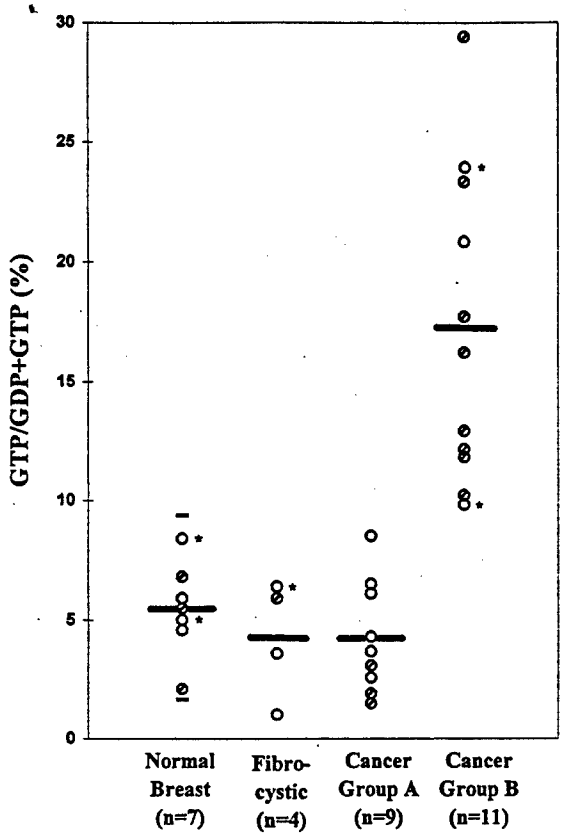


Fig. 1. Ras Activation Levels in Normal Breast Tissue, Fibrocystic Tissue, and Breast Cancers. Ras activation levels were measured as described in the text and are expressed as the ratio of GTP over GTP + GDP. Cancers Group A and B are defined in the text. The thick horizontal bar depicts the mean of each group; the two small horizontal bars in the normal group show two standard deviations beyond the mean of the group.

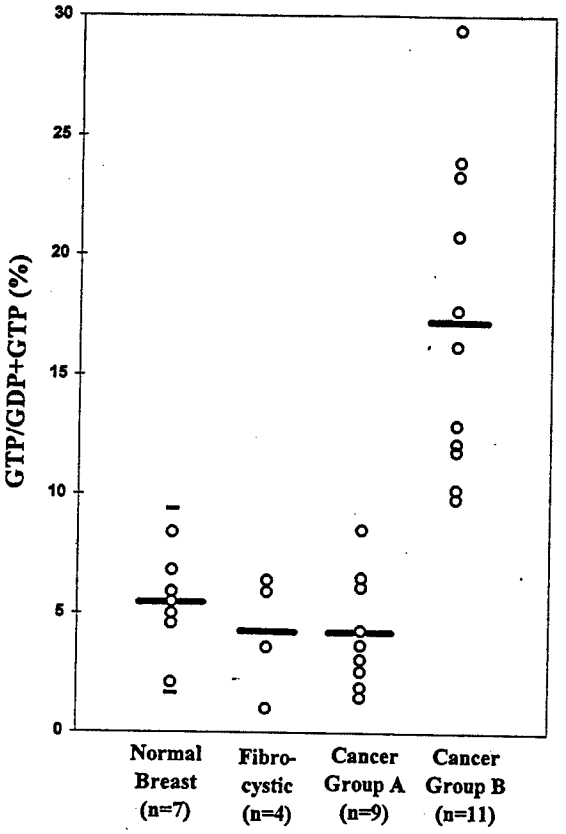
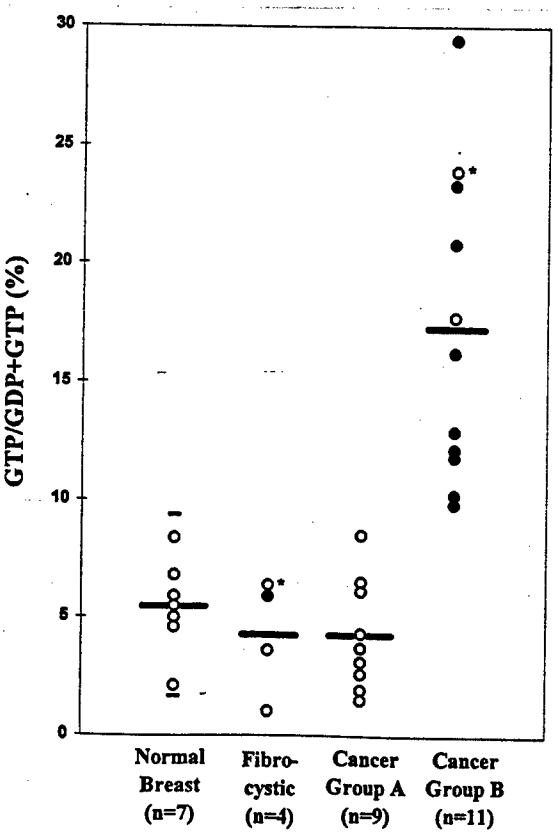


Fig. 2. EGF Receptor Positive Samples in the Study Cohort. These are the same data as in Fig. 1 but with the samples that were positive for the EGF receptor shown as filled circles; the asterisks depict two samples out of the entire group for which there was insufficient material to stain for the EGF receptor.

Fig. 3. ErbB-2 Receptor Positive Samples in the Study Cohort. These are the same data as in Fig. 1 but with the samples that were positive for the ErbB-2 receptor shown with a diagonal line through the circle; the asterisks depict five samples for which there was insufficient material to stain for the ErbB-2 receptor.



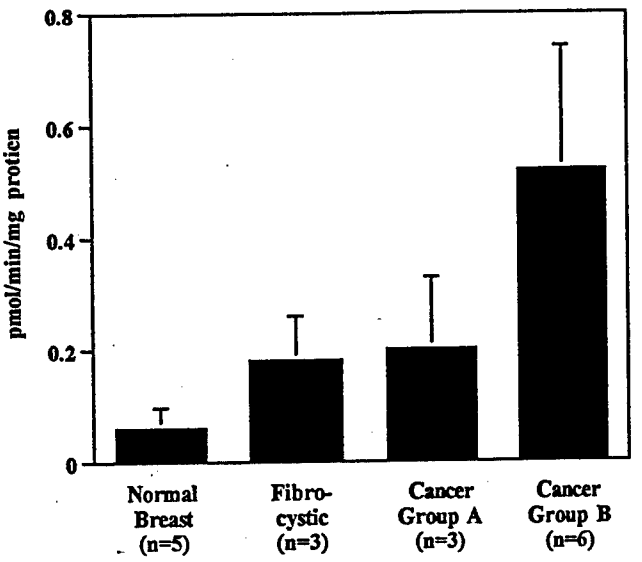


Fig. 4. MAP Kinase Activity in the Study Cohort. MAP kinase activity was measured as described in the text and is shown for normal breast tissue, fibrocystic breast tissue, and the two groups of breast cancers.

CONCLUSIONS

Although the sample size to date is small, we have found Ras to be significantly activated in at least half of breast cancers and the increased Ras activation correlates with expression of the EGF receptor. The increased Ras activation in the tumors appears to be of physiological significance since in those tumors with high Ras activation levels, MAP kinase activity was also elevated. Thus, the data suggest that our original hypothesis, "that Ras will be highly activated in a significant percentage of breast cancers because of overexpression of a growth factor receptor," appears to be correct. Clearly, more samples need to be analyzed and, at the present rate, we will analyze at least as many as outlined in the application's Statement of Work. The work performed in this grant will lay the groundwork for the future treatment of breast cancer with Ras inhibitors or other agents which inhibit signal transduction pathways in which Ras is involved.

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Ras-Activation in Human Breast Cancer correlates with Epidermal Growth Factor expression and activation of MAP kinase

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Abbreviations:

ABSTRACT

Background. The role of the human *ras* proto-oncogene and activation of the Ras/MAP kinase pathway in human cancers are under close investigation. Mutations in the *ras* gene are known to occur in over 90% of pancreatic cancers, 50% of colon cancers, 50% of thyroid cancers and ~5% of breast cancers. Although mutations in the *ras* gene are infrequent in breast cancers, there is considerable evidence that suggests that the Ras/MAP kinase pathway may still be deregulated and pathologically activated in breast cancer cells because different growth factor receptors which signal through the Ras and MAP kinase are overexpressed in a number of breast cancers. We therefore measured for the first time activation levels of Ras in benign and malignant human breast tissue samples using a new method which allowed us to determine Ras activation levels in vivo and without the use of radioactivity. We correlated the obtained activation levels with clinical and pathological data and studied possible activation mechanisms upstream and downstream of Ras.

Methods. We measured Ras activation levels using a recently described method ( ) for measuring absolute amounts of GTP and GDP bound to Ras in tissue samples from the following patient cohort: Seven patients with normal breast tissue, four patients with fibrocystic disease, one patient with fibroadenoma and 20 breast cancer patients. The obtained Ras activation levels in the patient cohort were compared to clinical and pathological data. All samples were assesed for mutations in the *ras* gene using the polymerase chaine reaction (PCR) and restricion fragment length polymorphism (RFLP), expression of the EGF, ErbB-2, c-FMS and truncated EGF receptors by immunohistochemistry, and activation of the MAP kinase downstream of Ras. The results were correlated with results obtained for three different human breast cancer cell lines: one "normal" epithelial breast cancer cell line, a cell line with overexpression of the ErbB-2 receptor and a cell line with a mutation in K-*ras*, codon 12.

Results. Of the 20 breast cancer samples nine had a mean Ras activation level of 4.2%, comparable to mean Ras activation levels of 5.5% and 4.4% for normal breast tissue and fibrocystic tissue and fibroadenoma, respectively. 11 of the 20 cancer samples had three-fold higher Ras activation levels with a mean of 17%. None of the breast cancer samples had a mutation in the *ras* gene. We found a remarkably good correlation between Ras activation levels and expression of the EGF receptor in the patient cohort: All samples with high Ras activation levels expressed the EGF receptor but none of the samples with normal Ras activation levels and none of the normal breast tissues. We found no similar significant correlation between high Ras activation and expression of the ErbB-2 receptor. The activity of the MAP kinase downstream of Ras was 2.5-fold increased in the breast cancers with high Ras activity compared to samples with normal Ras activation levels. These result were consistent with results obtained for the three different human breast cancer cell lines studied.

Conclusions. In our study we found considerable evidence that Ras/MAP kinase pathway is pathologically activated in slightly more than half of the 20 cancer samples investigated due to expression of the EFG receptor which is known to signal through the Ras/MAP kinase pathway. As none of the samples had an activating mutation in the *ras* gene, our results confirm the importance of upstream mechanisms for activation of Ras and MAP kinase and a possible role of EGF receptor expression in tumor cells and the developement of breast cancer. The pathological activation of the Ras/MAP kinase pathway makes these tumors a potential target for a new treatment with farnesyltransferase inhibitors which are known to specifically block downstream signaling of Ras.

INTRODUCTION

Breast cancer is one of the most common malignancies affecting 1 of 10 women in the United States and Western Europe during their lifetimes (Wooster et al., 1995) and respresenting about 25% of all female cancers. The etiology and pathogenesis of breast carcinoma remain unclear, but as is the case in other cancers, breast cancer appears to arise through a multistep process involving multiple genetic alterations (Christofori and Hanahan, 1994; Devilee and Cornelisse, 1994; Fearon an Vogelstein, 1990). Changes in the function of oncogenes and tumor suppressor genes through gene amplification, mutation, deletion, chromosomal rearrangement or translocation are among the common and important mechanisms (Jones et al., 1995). One of the oncogenes involved in the etiology and pathogenesis of many human cancers is the *ras* protooncogene and activation of the Ras/mitogen activated protein (MAP) kinase pathway stimulates cellular proliferation and differentiation and many cytokines and growth factors acitvate this pathway in cultured cells. A pathological activation of Ras and the MAP kinase pathway can lead to uncontrolled proliferation of cells and to malignant transformation and occurs after mutation in amino acids 12, 59 and 61 of Ras. These mutations lead to a loss of the regulatory GTPase-activity by which Ras is inactivated. Mutations in Ras are found in a variety of human cancers at varying percentages, including pancreatic cancer (95%), colon cancer (50%), thyroid cancer (50%) and breast cancer (5%) (for a review see Bos, J.L., 1984). The low percentage of breast cancers with Ras activation has led to the notion that Ras does not play a pathogenetic role in this cancer. However, several growth factor receptors which signal through the Ras/MAP kinase pathway are overexpressed in breast cancer suggesting that Ras may indeed be activated in a significant number of breast cancers. One of these growth factor receptors is the product of the c-*erb*B-2 proto-oncogene.

The c-*erb*B-2 proto-oncogene encodes a receptor tyrosine kinase (RTK) closely related to the epidermal growth factor receptor (EGFR). Overexpression of *erb*B-2 occurs in approximately 20 or more percent (reportet are expression levels varying from 20 to >40%, lit....) of human breast tumors and an increased expression correlates with poor patient prognosis. The EGFR is coupled to the Ras signalling pathway by interaction with the adaptor protein Grb2 (growth factor receptor binding protein), and SOS (son of sevenless), a Ras GDP/GTP exchange factor. Both, the EGF and the *erb*B-2-receptors belong to a class of membrane receptors with intrinsic tyrosine kinase activity. Growth factor binding to such receptor tyrosine kinases leads to receptor dimerization, activation of kinase activity and autophosphorylation on tyrosine residues, thus creating specific binding sites for signalling proteins containing src homology (SH) 2 domains (Schlessinger and Ullrich, 1992, Schlessinger, 1993).

In this study we investigated the activation state of the Ras and MAP kinase in 20 human breast cancer samples.

MATERIALS AND METHODS

Harvesting of Human Breast Tissue For Ras Activation Levels

Human breast tissue was obtained according to a procedure approved by the UCSD Institutional Review Board. For measurement of the Ras activation state, the pathologist came to the operating room at the time of tissue resection, sliced the specimen and applied cells adherent to the scalpel blade to glass slides which were placed on a block of dry ice. This method rapidly freezes the cells and yields >90% epithelial cells as determined by both histochemical and immunological staining (Figures 3A-D).

Measurement of Ras Activation State

The activation state of Ras is defined as the percent of Ras molecules in the active GTP-bound state, i.e., [Ras-bound GTP/Ras-bound GDP + Ras-bound GTP] X 100, and was measured in cells and tissue as previously described with the changes noted below (Scheele et al., 1995, Guha et al., 1996 and Guha et al., 1997). Briefly, frozen tissue was homogenized (by Dounce homogenization) in an ice-cold Hepes-based buffer containing 1% Nonidet P-40 with protease inhibitors and the resulting suspension shaken for 10 min at 4°C; for frozen cells, the homogenization step was omitted and the cells were resuspended in the Hepes-based buffer prior to shaking. From each sample 15 µl of the cell eluate was saved for the measurement of DNA (see below). The lysed cells were centrifuged at 10,000 X g for 5 min and to the supernatant was added NaCl, sodium dodecyl sulfate and deoxycholate to final concentrations of 500 mM, 0.05% and 0.5%, respectively. Half of the supernatant was added to protein G agarose beads (Gibco BRL, Grand Island, NY) preincubated with the rat monoclonal anti-Ras antibody Y13-259 (Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit secondary antibody (anti rat IgG-Fc, Cappel, Durham, NC) and the other half of the sample was added to beads preincubated with rat IgG and

the secondary antibody. The samples were shaken gently for 1 h at 4°C and the immunoprecipitates were washed X 4 in a detergent-based buffer and X 2 in a Tris-based buffer. The washed immunoprecipitates were resuspended in a TrisPO_4/EDTA/dithiothreitol solution and heated to 100°C for 3 min to elute GTP and GDP from the immunoprecipitated Ras; we have shown by HPLC that at neutral pH less then 5% of GTP is destroyed when heated to 100°C for up to 10 min (Scheele et al., 1995).

GTP was measured by converting it to ATP using the enzyme nucleoside diphosphate kinase (NDPK, Sigma, St. Louis, MO) and ATP was measured by the luciferase/luciferin system according to the following reactions.

Reaction 1: NDP kinase

$$GTP + ATP \rightarrow GDP + ATP$$

Reaction 2: luciferase

$$GTP + luciferin \rightarrow oxyluciferin + AMP + PPi + light$$

where PPi is pyrophosphate. This assay is sensitive to 1 fmol of GTP and was performed as described previously except the sample was preincubated for 30 min with all components of the system excluding the NDPK; this preincubation removes ATP or any other cellular component contaminating the samples which could cause light generation, and thereby lowers the background signal.

GDP was measured by converting it to GTP using pyruvate kinase (ICN, Costa Mesa, CA) and phosphoenolpyruvate (Sigma, St. Louis, MO) with the GTP measured as described above:

Reaction 3: pyruvate kinase

$$GDP + phosphoenolpyruvate \rightarrow GTP + pyruvate$$

Because the final product is again emitted light, this assay is also sensitive to 1 fmol (Sharma P.M. et al., 1998). When GTP is measured in the second step the sum of GTP + GDP is determined; thus, the amount of GTP in the sample must be substracted from the amount of GTP + GDP to yield the amount of GDP.

The amounts of GDP and GTP in the samples were determined from standard curves prepared with each set of samples and the data are expressed as fmol of GTP or GDP per microgram DNA.

Assessment for Activating Mutations in the *ras* Gene by Polymerase Chain Reaction (PCR)

DNA was isolated from frozen or paraffin-embedded tissue and from frozen cell pellets. The paraffin embeded tissue was sliced in 8 μm pieces and the paraffin was dissolved using xylene with the released tissue washed X 2 in 100% ethanol. Frozen tissue and cell pellets were quickly homogenized (using a Dounce homogenizer for the tissue) in a lysis buffer (Puregene, Minneapolis, MN) and DNA isolation was performed using a Puregene kit (Puregene, Minneapolis, MN). Activating mutations in the *ras* gene were detected according to Jiang et al., 1989, using at least 400 ng DNA per sample. As a positive control, human pancreatic cancer tissue with a mutation in K-*ras,* codon 12, was used. As a negative controle (wild type *ras*) the human breast cancer cell line MCF-7 was used. An aliquot of the PCR reaction was analyzed by agarose gel electrophoresis and ethidium-bromide staining to assure that sufficient product was generated. One half of the PCR product was then digested with the restriction enzyme BstNI (BioLabs, New England) and the other half served as a control. After BstNI digestion, the samples were analyzed by 10% polyacrylamide gel electrophoresis and ethidium-bromide staining. A negative and a positive control were included with all samples.

Immunohistochemistry

Frozen sections were fixed in 10% buffered formalin and overlayed with the primary antibodies. A rabbit anti-EGFR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-human ErbB-2 polyclonal antibody with cross-reactivity to mouse (Dako Co., Carpinteria, CA), a mouse anti-human truncated EGFR polyclonal antibody (provided by) and a rabbit anti-keratin polyclonal antibody (Dako Co., Carpinteria, CA) were used. Binding was detected using the DAKO's alkaline phosphatase LSAB kit following manufacturer's instructions. The tissue sections were developed using the Vector Blue substrate and counterstained with Nuclear fast red.

MAP Kinase Assay

Mitogen-activated protein (MAP) kinase activity was measured following phosphorylation of myelin basic protein (MBP) in MAP kinase immunoprecipitates (Whitehurst et al., 1995). Briefly, frozen cell pellets or tissue samples were extracted as described for immunoprecipitating Ras and a rabbit polyclonal anti-MAP kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the cell lysate along with protein G agarose beads. After a 1 h incubation at 4°C and gentle shaking, the resulting MAP kinase immunoprecipitates were washed and then incubated with MBP (Sigma, St. Louis, MO) and [γ-^{32}P]ATP (DuPont, Wilmington, DE) for 10 min at 30°C. The reaction was stopped by spotting an aliquot of the mix on P81 ion exchange chromatography paper and the papers were washed in dilute phosphoric acid. The papers were dried and bound radioactivity representing phosphorylated MBP was measured in a liquid scintillation counter. The data are expressed as pmol/min/mg protein and the assay was linear with time and protein concentration.

Cell Culture

The human breast cancer cell lines MCF-7, MDA-MB-453 and MDA-MB-231 were obtained from the American Type Culture Collection (Rockville, MD). Cultures were maintained at 37°C, 5% CO_2 in 10 cm diameter cell culture dishes and cells were grown in DMEM/F12 medium (Gibco BRL, Grand Island, NY) containing 8% transferrin-enriched calf serum (ECS, Gemini Bioproducts Inc., Calabasas, CA) and 2% fetal calf serum (FCS, Gibco BRL, Grand Island, NY). Cells were harvested in mid-logarithmic phase for measurement of the Ras activation state (~10 X 10^6 cells per sample) and MAP kinase activity and for Western blots. For growth factor stimulation experiments, cells were starved in mid-logarithmic phase for 72 h in DMEM-F12 without serum. Cells were then incubated for 5 min with DMEM/F12 containing either 20% FCS or 100 ng/ml epidermal growth factor (EGF), washed X 1 with ice-cold PBS and harvested by centrifugation. Cell pellets were immediately frozen on dry ice and kept at -80°C until use.

Measurement of DNA and Protein

DNA was measured in tissue and cell lysates by a standard fluorescence method using the fluorescent dye bisbenzimidazole (Brunk et al., 1979) and protein was measured by the Bradford method (Bradford et al., 1976).

Statistical Evaluation of Data

RESULTS

Clinical and Pathological Data

The clinical and pathological data of the patient cohort are shown in Tables IA-IC. Included in these tables are the Ras activation measurements to allow direct comparison between the data in these tables and the data presented later in Figures 1A-C.

We obtained normal breast epithelial cells from seven subjects ranging in age from 33 to 68 (Table IA). In ....patients, the breast tissue was obtained as part of a reduction mammoplasty and in ....patients, the tissue was obtained during a prophylactic mastectomy because of a history of breast cancer in the contralateral breast. In patient 2, a mastectomy was performed for breast cancer and normal epithelial cells distant from the cancer and cells from the cancer itself were analyzed. Included in our study were three patients with fibrocystic disease and one patient with a fibroadenoma. In patient 9, a mastectomy was performed for breast cancer and, as in patient 2, the fibrocystic tissue distant from the cancer and the cancer tissue itself were analyzed.

We analyzed breast cancers from 20 patients and divided these patients into two groups based on their Ras activation levels (as discussed below). There were no significant differences in the clinical or pathological data between the two groups. Specifically, their age range and clinical stage were similar as were the size, grade and histology of the tumors (Tables IB and IC). In addition, the percent of cells in S phase and the chromosomal ploidy of the tumors was similar in the two groups and the estrogen and progesterone receptor status was also similar in both groups. Thus, the two groups were indistinguishable by standard clinical and laboratory parameters.

Ras Activation Levels in Human Breast Tissue

Ras activation levels in the samples from all patients described in Tables IA-IC are shown graphically in Fig. 1. In the seven normal breast tissue samples, Ra

s activation levels varied between 2.1 and 8.4% with a mean of 5.5%; Ras activation levels within two standard deviations of the mean value of these seven normal samples were from 1.6 to 9.4%. These values are similar to what we have found in other normal human tissues and cells, including brain, peripheral nerve, peripheral blood leukocytes and ovarian epithelial cells (Guha et al., 1996, Guha et al., 1997 and unpublished data). In samples from patients with fibrocystic disease and fibroadenoma, the distribution of Ras activation levels was similar to normal breast tissue, varying between 1.0 and 6.4% with a mean of 4.2%.

When we measured Ras activation levels in the 20 breast cancers, we found that the activation levels varied between 1.5 and 29% (Tables IB and IC and Fig. 1). Of the 20 cancers, nine had Ras activation levels within the range of two standard deviations of the mean value obtained in the normal breast samples and these nine breast cancer samples were combined together as Group A in Table IB and Fig. 1. For 11 of the cancers, or slightly more than half of the total, Ras activation levels exceeded two standard deviations above the mean of the normal samples and these cancers were combined together as Group B in Table IC and Fig. 1; the mean Ras activation level for these 11 cancers was 17%, or more than three times the mean Ras activation level of the normal breast samples. The elevated Ras activation levels in these 11 cancers could not be attributed to a generalized increase in Ras activation levels in these patient's breast tissue because Ras activation levels in normal breast tissue and fibrocystic tissue distant from the tumors of patients 2 and 9, respectively, were within the normal range (compare Tables

IA and IC). As mentioned earlier, there were no discernible differences between the clinical and pathological data of the patients in Groups A and B.

Assesement for Activating Mutations in the *ras* Gene by Polymerase Chain Reaction (PCR)

The increased Ras activation levels we found in 11 of the 20 breast cancers could be secondary to increased Ras activation due to a mutation in the *ras* gene. As mentioned earlier, mutations are found in approximately 5% of human breast cancers. We therefore assesed the breast cancer samples for activating mutations in the *ras* gene by polymerase chain reaction (PCR). As a control for wild type *ras* (negative control) we used the human epithelial breast cancer cell line MCF-7, as a positive control for a mutation in K-*ras*, codon 12, we used human pancreatic cancer tissue. PCR amplification of K-*ras* first exon segments using K5' and K3' as primers (Jiang W. et al., 1989) generated a DNA fragment of 157 nucleotides, visible by native polyacrylamide gel electrophoresis and ethidium bromide staining. Upon incubation with the restriction enzyme BstNI, fragments encoding wild type codon 12 sequences were cleaved twice, resulting in a band of 114 nucleotides (and smaller bands of 29 and 14 nucleotides). Fragments containing a mutation at either the first or second position of codon 12 were cleaved only once, resulting in a larger band of 143 nucleotides (and a band of 14 nucleotides). Fig. 2 shows a representative polyacrylamide gel after ethidium bromide staining. The cleavage pattern for wild type *ras* was detectable in the "normal " epithelial breast cancer cell line MCF-7 (Fig. 2, lane 1A and B), the cleavage pattern for a mutation in K-ras, codon 12, was detectable in the pancreatic cancer tissue (Fig. 2, lane 2A and B). Lanes 3 and 4 show two samples from Group A breast cancers (patients 12 and 17) and lanes 5 to 7 three samples from Group B breast cancers (patients 9, 27 and 29). None of these samples contained mutations at either position of codon 12. This result is representative for the study cohort: In none of seven tissue samples from Group A breast cancers (patients 12, 13, 14, 16, 17,

18 and 19) and in none of eight samples from Group B breast cancers (patients 21, 22, 23, 24, 9, 2, 27 and 29), including samples with Ras activation levels higher than 20% , we could detect a mutation in K-ras (no result could be obtained for patients 15 and 20 from Group A and patients 25, 26 and 28 from Group B). As expected, the normal breast tissues and the fibrocystic tissues had wild type *ras* (data not shown).

Expression of the EGF, ErbB-2 (HER2/*neu*) and c-FMS Receptors in the Patient Cohort

As none of the breast cancer samples in the study cohort had shown a mutation in the *ras* gene, the increased Ras activation levels could be secondary to increased Ras activation because of overexpression of the EGF, ErbB-2 or c-FMS receptors in these cancers. All three of these growth factor receptors relay growth promoting signals to the nucleus via the Ras/MAP kinase pathway.

We, therefore, analyzed tissue samples from each patient by immunohistochemical staining for expression of the EGF, ErbB-2 and c-FMS receptors. All samples were assesed in a blinded fashion (without knowledge of the Ras activation levels in the samples) by two independent pathologists. A representative picture of what was defined as a positive result for EGF and ErbB-2 receptor overexpression is shown in Figure 3A and B together with a negative control (Figure 3C) along with immunostaining for alpha keratin (Figure 3D).

Figs. 4A and 4B show the results for EGF and ErbB-2 receptor expression, respectively. All normal breast tissue samples (Fig. 4A) were negative for expression of the EGF receptor. One sample (patient 9) from the patients with fibrocystic disease showed a positive result; this fibrocystic tissue was obtained from a patient operated for breast cancer and the cancer tissue also showed high expression of the EGF receptor. None of the samples in breast cancer Group A (column 3) with normal Ras activation levels expressed the EGF receptor. In contrast, all samples but one (patient 26, see Table IC), in the breast cancer Group B, samples with high Ras activation

levels, expressed the EGF receptor (for patient 28 a result was not available). Thus, in nine of ten breast cancers with high Ras activation, the EGF receptor was expressed whereas the EGF receptor was not expressed in any of the nine breast cancers with low Ras activation levels nor in any of the seven normal breast tissues; the only outlier was patient 9 in whom the EGF receptor was expressed in nonmalignant fibrocystic tissue, but this patient had a carcinoma which expressed the EGF receptor.

The data with the EGF receptor are in contrast to what we found when we assessed ErbB-2 receptor expression. Three of the normal breast tissue samples expressed the ErbB-2 receptor (patients 1, 4 and 6) with patient 1 having an adenocarcinoma in the contralateral breast. One of the patients with fibrocystic tissue, patient 9 who was operated for cancer, expressed both the EGF and the ErbB-2 receptors in the non-malignant tissue. In the Group A breast cancers three of nine samples or 33% expressed the ErbB-2 receptor (patients 12, 13 and 15, see Table IC and Fig. 4B). In breast cancer Group B one sample was negative for ErbB-2 expression (patient 2, see Table IC and Fig. 4B) and results could not be obtained for two patients (patients 21 and 28, see Table IC). All the other samples in Group B stained positive for the ErbB-2 receptor (patients 22, 23, 24, 9, 25, 26, 27 and 29, see Table IC and Fig. 4B).

Thus, there was a remarkably good correlation between expression of the EGF receptor and Ras activation levels in this series of breast cancers while there was a much lesser correlation between Ras activation levels and expression of the ErbB-2 receptor. Interestingly, the one cancer with high Ras activation levels which did not express the EGF receptor expressed the ErbB-2 receptor. None of the samples, either normal or malignant, expressed the c-FMS receptor nor did any of the samples express a truncated constitutively-active EGF receptor.

Activity of MAP Kinase in Human Breast Tissue

One of the major downstream effectors activated by Ras is MAP kinase. Having shown that the EGF receptor was expressed in the Group B breast cancers, but not in the Group A breast cancers, we determined the activity of MAP kinase in a representative number of samples in both study groups. This allowed us to determine if a high Ras activation level correlated with a high activation state of MAP kinase.

Fig. 5 shows the results for MAP kinase activity in five normal breast tissue samples, three fibrocystic tissue samples, three samples from the Group A breast cancers, and six samples from the Group B breast cancers. The normal breast tissues, fibrocystic tissues and the Group A breast cancers had MAP kinase activities of 0.06 ± 0.04, 0.18 ± 0.08 and 0.20 ± 0.13 pmol/min/mg protein, respectively. Because of the rather high standard deviations, there was no significant difference in the MAP kinase activity among these three tissue types. The MAP kinase activity of the Group B breast cancers was 0.52 ± 0.22 pmol/min/mg protein, which was significantly higher than in any of the other three tissue types (two-tailed Student test). Therefore, the samples with high Ras activation levels also showed high MAP kinase activity and the three-fold higher Ras activation in the Group B breast cancers compared to the Group A cancers was reflected in a 2.5-fold increase in MAP kinase activity.

Ras Activation Levels and MAP kinase activity in the Human Breast Cancer Cell Lines

Human cell lines are a well established and valuable model system for the investigation of cellular functions in a homogeneous cell type and the results obtained from *in vitro* experiments allow comparison with physiological or pathological conditions *in vivo*.

To compare the results obtained from the samples of patients in the study cohort to cultured breast cancer cells, we tested three different human breast cancer cell lines for their Ras activation

levels under the following conditions: cells grown in culture medium in mid-logarithmic phase, cells under serum starvation, and serum-starved cells after growth factor stimulation using fetal calf serum (FCS) or epidermal growth factor (EGF). The three different human breast cancer cell lines studied were a "normal" epithelial breast cancer cell line (MCF-7), a cell line overexpressing the ErbB-2 receptor and a cell line with a mutation in amino acid 12 of K-Ras. Under normal growth conditions, MCF-7 cells had a mean Ras activation level of 4.5% (Fig. 6). This Ras activation level was comparable to the mean activation level obtained in the Group A breast cancers of the patient cohort (see Fig. 1A). Under serum starvation, the activation levels decreased to 2%, addition of FCS or EGF led to an approximate 4-fold increase in Ras activation with no significant difference between FCS or EGF stimulation. The breast cancer cell line which overexpressed the ErbB-2 receptor (MDA-MB-453) showed a much higher Ras activation level under normal growth conditions (mean 17%, Fig. 6), comparable to the values obtained from breast cancer Group B (see Fig. 1A). Under serum starvation this value decreased to 8%, and addition of FCS and EGF increased the Ras activation level ~1.5-fold and 3.5-fold, respectively. The cell line with a mutation in amino acid 12 in K-Ras had a high Ras activation level of ~30% under normal growth conditions and in contrast to both the MCF-7 and the MDA-MB-453 cell lines, the Ras activation level in this cell line was not significantly changed under serum starvation or growth factor stimulation.

We also measured MAP kinase activity in the cell lines (Fig. 7) and found a value of 0.8 pmol/min/mg protein under serum starvation for cell line MCF-7 which increased ~3.6-fold with EGF. MAP kinase activity in MDA-MB-453 cells was higher under serum starvation (0.25 pmol/min/mg protein) compared to the MCF-7 cell line and increased ~1.8-fold after stimulation

with EGF. In contrast, MAP kinase activity in the cell line with the mutation in K-Ras again was not influenced by growth factor stimulation.

DISCUSSION

In this study we measured the Ras activation levels in seven normal breast tissue samples, three samples from patients with fibrocystic disease, one fibroadenoma and 20 breast cancer samples. We found that Ras activation levels in normal breast tissue varied between 2.1 and 8.4% and that Ras activation levels within two standard deviations of the mean value of these seven normal samples were from 1.6 to 9.4%. These values were similar to what we have found in other normal human tissues and cells, including brain, peripheral nerve, peripheral blood leukocytes and ovarian epithelial cells (Guha et al., 1996, Guha et al., 1997 and unpublished data). The distribution level of Ras activation was similar in samples from patients with fibrocystic disease and fibroadenoma. Of the 20 breast cancers, nine had Ras activation levels within the range of two standard deviations of the mean value obtained in the normal breast cancer samples (Group A). The mean Ras activation level of 11 cancer samples was 17% or more than 3 times the mean of Ras activation level of the normal breast samples (Group B).

As these increased Ras activation levels in the Group B breast cancers could have been secondary to increased Ras activation due to a mutation in the *ras* gene, we assesed the breast cancer samples for activating mutations by polymerase chain reaction, using the human epithelial breast cancer cell line MCF-7 as a control for wild type K-*ras* and human pancreatic cancer tissue as a positive control for a mutation in K-*ras*, codon 12. Our studies were limited to codon 12 of the K-*ras* gene, since mutations were described to occure preferentially in this codon (Yanez, L. et al., 1987, Koffa, M. et al., 1994). We found neither in tumor samples with normal nor in cancer

samples with high Ras activation levels mutations in K-*ras*, codon 12 , and - as expected - none of the normal or the fibrocystic tissues had a *ras* mutation.

As the increased Ras activation levels could be secondary to overexpression of the EGF, ErbB-2 or c-FMS receptors in these cancers, we analyzed tissue samples from each patient by immunohistochemical staining for expression of the EGF, ErbB-2 and c-FMS receptors. We found a remarkably good correlation between expression of the EGF receptor and Ras activation levels in the study cohort: All normal breast tissues were negative for expression of the EGF receptor and none of the samples from breast cancer Group A with normal Ras activation levels expressed the EGF receptor. In contrast, all samples but one in the breast cancer Group B, samples with high Ras activation levels, expressed the EGF receptor. The only outlier was patient 9 in whom the EGF receptor was expressed in nonmalignant fibrocystic tissue, but this patient had a carcinoma which expressed the EGF receptor.

These data were in contrast to what we found when we assesed ErbB-2 receptor expression, where we found a much lesser correlation between Ras activation levels and receptor expression. None of the samples, neither normal or malignant, expressed the c-FMS receptor nor did any of the samples express a truncated constitutively-acitve EGF receptor.

Because one of the major downstream effectors activated by Ras is MAP kinase, we determined, if the high Ras activation levels correlated with a higher activation state of MAP kinase. We found that the three-fold higher Ras activation in the Group B breast cancers compared to Group A was reflected in a 2.5-fold increase in MAP kinase activity.

In the presented study we measured for the first time Ras activation levels in human breast cancer tissues with a recently described method for measuring absolute amounts of GTP and GDP bound to Ras (1223).

Figure Legends

Figure 1: Ras Activation Levels in Cohort Group

Figure 2: PCR for K-*ras*, codon 12

Figure 3: Immunohistochemistry for EGF and ErbB-2 Receptors

Figure 4: EGF and ErbB-2 Receptors Expression in Cohort Group

Figure 5: MAP Kinase Activity in Cohort Group

Figure 6: Ras Activation Levels in Breast Cancer Cell Lines

Figure 7: MAP Kinase Activity in Human Breast Cancer Cell Lines

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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